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Anaerobic Mineralization of Quaternary Carbon Atoms: Isolation of Denitrifying Bacteria on Pivalic Acid (2,2-Dimethylpropionic Acid)

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The degradability of pivalic acid was established by the isolation of several facultative denitrifying strains belonging to *Zoogloea resiniphila*, to *Thauera* and *Herbaspirillum*, and to *Comamonadaceae*, related to [*Aquaspirillum*] and *Acidovorax*, and of a nitrate-reducing bacterium affiliated with *Moraxella osloensis*. Pivalic acid was completely mineralized to carbon dioxide. The catabolic pathways may involve an oxidation to dimethylmalonate or a carbon skeleton rearrangement, a putative 2,2-dimethylpropionyl coenzyme A mutase.

Quaternary carbon atoms bind with all four single σ bonds to carbon atoms. This structural motif is present in natural compounds as well as in xenobiotic substances. Resin acids, tricyclic diterpenes, are present in cell walls and are discharged during the pulping process, yielding toxic wastewaters. Aerobic growth of bacteria on defined resin acids has been studied to the molecular level: a dioxygenase initiates the degradation of dehydroabietic acid by *Pseudomonas abietaniphila* (13). The microbial degradation of compounds with quaternary carbon atoms often involves mono- and dioxygenases. Hence, the persistence of the compounds is expected to be particularly high in anaerobic habitats. So far, no pure cultures of anaerobic microorganisms which use resin acids as a source of carbon or energy have been obtained (14). In contrast, we previously isolated denitrifying bacteria on compounds with quaternary carbon atoms: cholesterol (10), dimethylmalonate (11), and monoterpenes (6, 7). For cholesterol and dimethylmalonate, we suggested that dimethylmalonyl coenzyme A (CoA) decarboxylases might convert the quaternary carbon to a tertiary carbon atom. Neither resin acids nor pivalic acid (2,2-dimethylpropionic acid) can undergo such a reaction because of a lack of a functional group in the β position. Pivalic acid does occur in nature (20). A biosynthetic pathway is not known, and it is most likely of anthropogenic origin: pivalic acid esters are established prodrugs. Pivalic acid was considered previously not to be biodegradable: it was and still is an established reference substance in the determination of volatile fatty acid production by rumen microorganisms (4, 5). However, pivalic acid in pharmaceutical wastewater was found to be biodegradable in a methanogenic upflow anaerobic biofilter process (3) and in the denitrifying stage of a sequencing batch reactor (17). Hence, we attempted the isolation of anaerobic bacteria on pivalic acid as the simplest β-nonfunctional–α-quaternary carboxylic acid.

Enrichment and isolation. Denitrifying bacteria are usually facultative anaerobes, capable of anaerobic nitrate and aerobic oxygen respiration. So far, of the many strictly anaerobically isolated nitrate-reducing bacteria only *Azoarcus anaerobius* is unable to respire oxygen (21). Our isolation strategy with pivalic acid as sole electron donor and carbon source aimed at this physiology in order to obtain many strains quickly: enrichment of an anaerobic nitrate-reducing population in liquid culture was followed by repeated aerobic growth on oxic plates to obtain single colonies. Isolated strains were tested for anaerobic growth on pivalic acid and nitrate. Anoxic media and culture techniques were applied as described previously (11).

A large inoculum (10 ml, 10% [vol/vol]) of activated sewage sludge from a local wastewater treatment plant (Lintel, Osterholz-Scharmbeck, Germany) was incubated with 10 mM pivalate and 10 mM nitrate. The electron acceptor was consumed within 5 days both in the presence and in the absence of pivalate, probably due to electron donors in the sludge. Nitrate consumption continued in the enrichment culture, and nitrate was replenished three times, before the enrichment was transferred to fresh denitrifying medium. After four transfers (inoculum of 10% [vol/vol]), a pure strain was obtained by isolation of colonies grown on oxic, nitrate-free agar plates with 10 mM pivalate. The isolated strain was named PIV-1 and maintained at the Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany, under number DSM 14691. A tiny inoculum of 0.2 ml of sewage sludge was the source of a second enrichment in 15-ml cultures. The enrichment culture was directly streaked on oxic pivalate plates. Two morphologically homogeneous colonies formed white and yellow colonies after repeated transfer onto plates. These strains were named PIV-3A2w (DSM 14766), PIV-3A2y, PIV-3C2w, and PIV-3C2y. A different colony type led to the isolation of strain PIV-3D (DSM 14692).

To obtain a broad collection of strains, 38 different soil and freshwater samples of 1 g or 1 ml in size, respectively, were collected near Bremen, Germany, in July 2000 and were incubated with pivalate and nitrate. Nitrate reduction occurred in 30 cultures. Transfer of a small inoculum (2 ml, 0.5% [vol/vol]) resulted in growth in 15 enrichment cultures. These were streaked out on oxic pivalate plates (30 plates). Colonies grown were transferred for purification on brain heart infusion agar: 28 of 30 strains grew. The physiological capacity to grow on pivalate was confirmed first on oxic pivalate plates (24 of 28 strains grew) and then in anoxic medium with pivalate and nitrate. Eight strains from six habitats were finally obtained:

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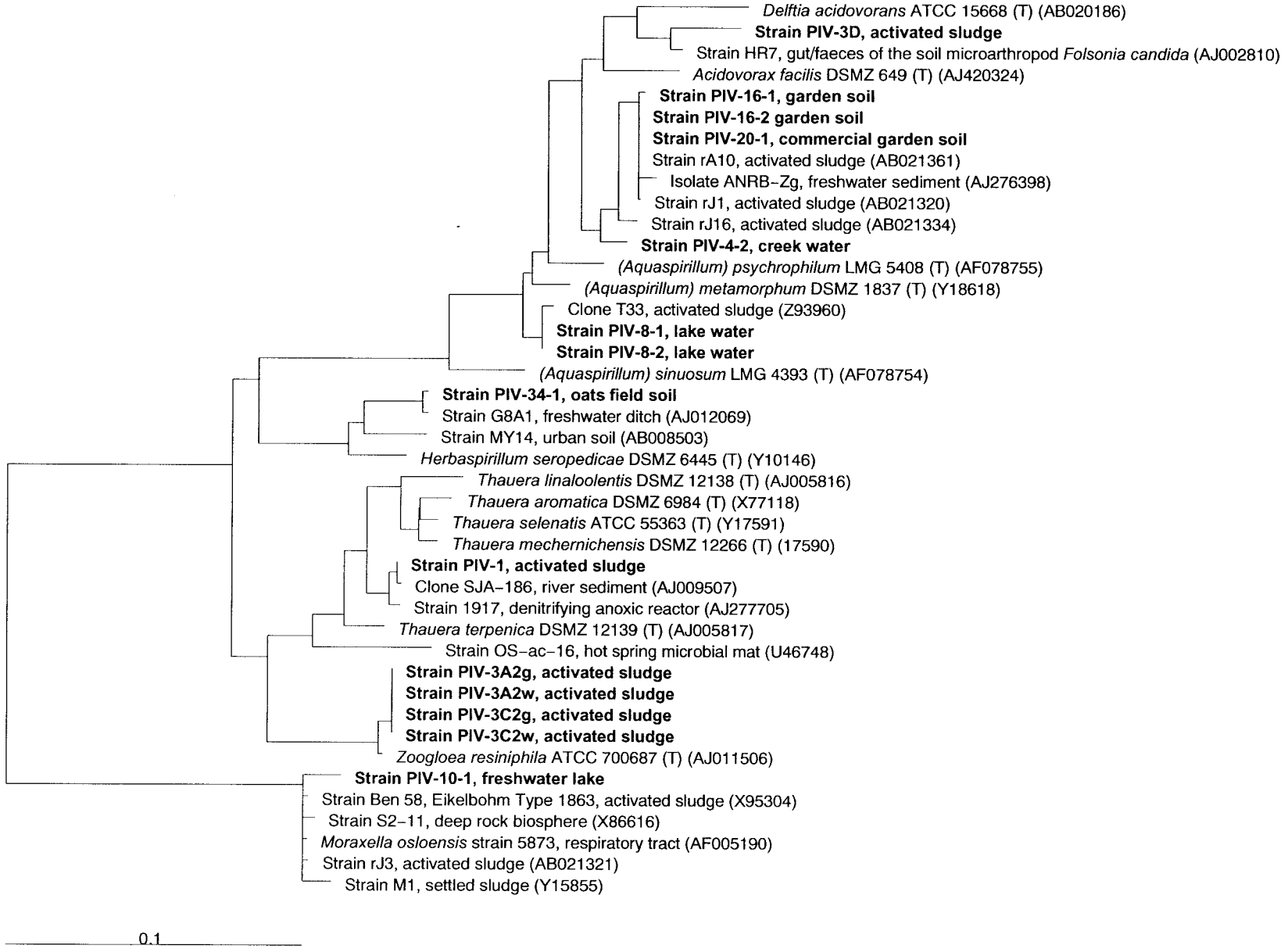

FIG. 1. 16S rRNA-based tree reflecting the phylogenetic relationships of strains isolated on pivalate. The tree is based on the results of a distance matrix analysis including complete or almost complete 16S rRNA sequences from representative bacteria of the beta- and gammaproteobacteria. The topology of the tree was evaluated and corrected according to the results of distance matrix, maximum parsimony, and maximum likelihood analyses of various data sets. Phylogenetic positions of the analyzed strains did not differ in any of the treeing approaches. Multifurcations indicate topologies that could not be unambiguously resolved. The bar indicates 10% estimated sequence divergence.

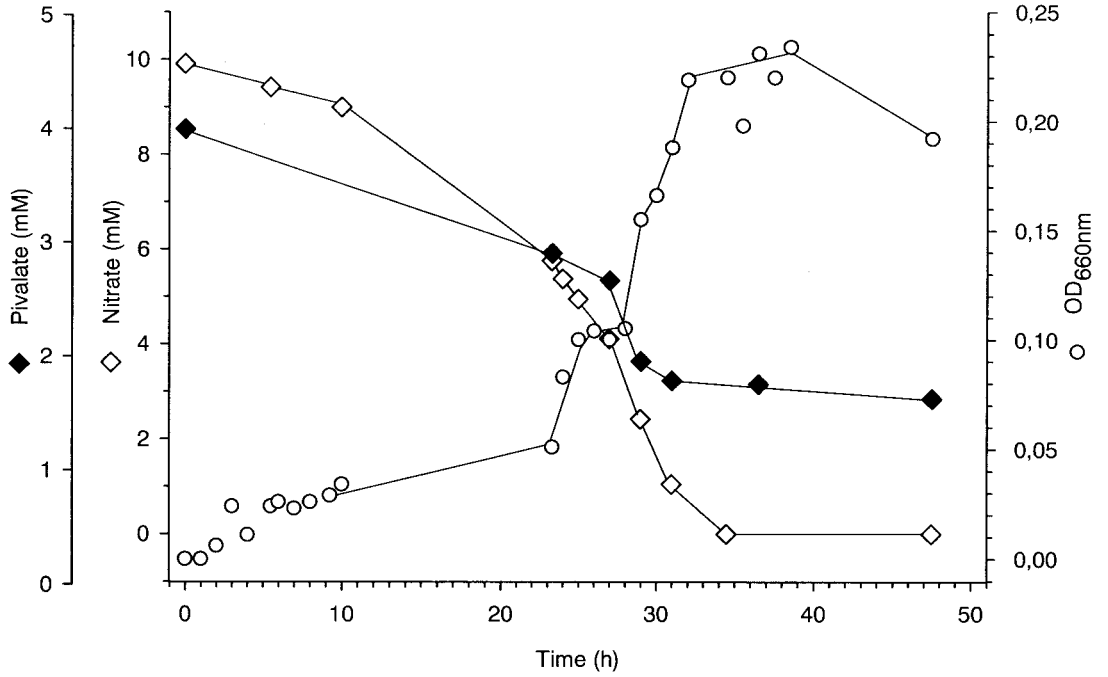

FIG. 2. Growth of strain PIV-1 on pivalate and nitrate.

PIV-4-2 (creek water, DSM 15240), PIV-8-1 (DSM 14767) and PIV-8-2 (river water), PIV-10-1 (lake water), PIV-16-1 and PIV-16-2 (garden soil), PIV-20-1 (commercial flower soil), and PIV-34-1 (oat field soil, DSM 14683). All strains denitrified, with one exception: strain PIV-10-1 reduced nitrate to nitrite. The strain did not grow after some transfers and was lost.

To gain initial insight into the ecological importance of the bacteria, we performed most probable number counts on the activated sewage sludge (1, 11) and found significant populations of aerobic and anaerobic bacteria growing on pivalate (24×10^3 and 4.6×10^3 cultivable cells ml^{-1}, respectively).

Phylogenetic affiliation. Almost-complete 16S rRNA gene sequences (over 1,450 nucleotides) were obtained by in vitro amplification and direct sequencing (16). By applying the ARB program package (12), the sequences were aligned and phylogenetic trees were constructed to elaborate a consensus tree (Fig. 1).

A broad diversity of betaproteobacteria was identified. Strain PIV-1 affiliated with the betaproteobacterial genus *Thauera* (97.7% 16S similarity to *Thauera selenatis*). Strains PIV-3A2w, PIV-3A2y, PIV-3C2w, and PIV-3C2y were also placed in the family *Rhodocyclaceae*; they belong to *Zoogloea resiniphila* (99.6% 16S similarity). Interestingly, the type strain of *Zoogloea resiniphila* was isolated as an aerobic resin acid-degrading strain with the capacity of denitrification on pyruvate (15). Three groups of strains are members of the family *Comamonadaceae*: strain PIV-3D is related to [*Aquaspirillum*] *psychrophilum*, *Acidovorax facilis*, and *Delftia acidovorans* (95.9, 95.7, and 95.7% 16S similarity, respectively). Strains PIV-4-2, -16-1, -16-2, and -20-1 are associated with [*Aquaspirillum*] *psychrophilum* within the *Comamonadaceae* (97.5 to 97.8% 16S similarity). Strains PIV-8-1 and PIV-8-2 are affiliated with [*Aquaspirillum*] *metanomorphum* (98.0% 16S similarity). A third family of betaproteobacteria is present: strain PIV-34-1 is a member of the *Oxalobacteraceae*, related to the genus *Herbaspirillum* (97.4% 16S similarity) and to strain G8A1, a dimethylmalonate-degrading denitrifying betaproteobacterium (99.7% 16S similarity) (11). The nitrate-reducing strain PIV-10-1 was allocated to a nonclassical *Moraxella* clade in a group of environmental clone sequences and *Moraxella osloensis* in the gammaproteobacteria (99.2% 16S similarity to *Moraxella osloensis*) (18).

Quantification of denitrifying growth on pivalate. Strain PIV-1 was used to determine quantitatively the consumption of pivalate in the presence of nitrate. Analytical methods were applied as described previously (7–10). A method for pivalic acid quantification was established that applied a gas chromatograph with a flame ionization detector and a BP20 polar phase column (0.32 mm by 50 m; 0.5-μm film thickness; SGE, Darmstadt, Germany). One-microliter samples were separated with hydrogen as carrier gas at 2 ml min^{-1} and the following temperature program: injection port, 250°C; column, 110°C for 2 min, increasing at a rate of 10°C min^{-1}; 200°C for 0.1 min, increasing at a rate of 40°C min^{-1}; 250°C for 5 min; flame detector, 280°C.

Under nitrate-limiting conditions, strain PIV-1 grew fast, without transient nitrite accumulation (Fig. 2). Complete consumption of 9.91 mM nitrate concurred with the disappearance of 2.37 mM pivalate. A biomass of 146 mg $liter^{-1}$ was formed based on a measured correlation of 633 mg (dry mass) $liter^{-1}$ to 1 liter of culture with an optical density at 660 nm of 1.0. According to the assimilation equation $17\ C_5H_{10}O_2 + 19\ CO_2 + 6\ H_2O \rightarrow 26\ C_4H_7O_3$, this corresponds to a consumption of 0.93 mM pivalate. Ammonia present in the medium was assimilated (2.6 mM). The complete reduction of nitrate to dinitrogen according to the dissimilation equation $5\ C_5H_{10}O_2 +$

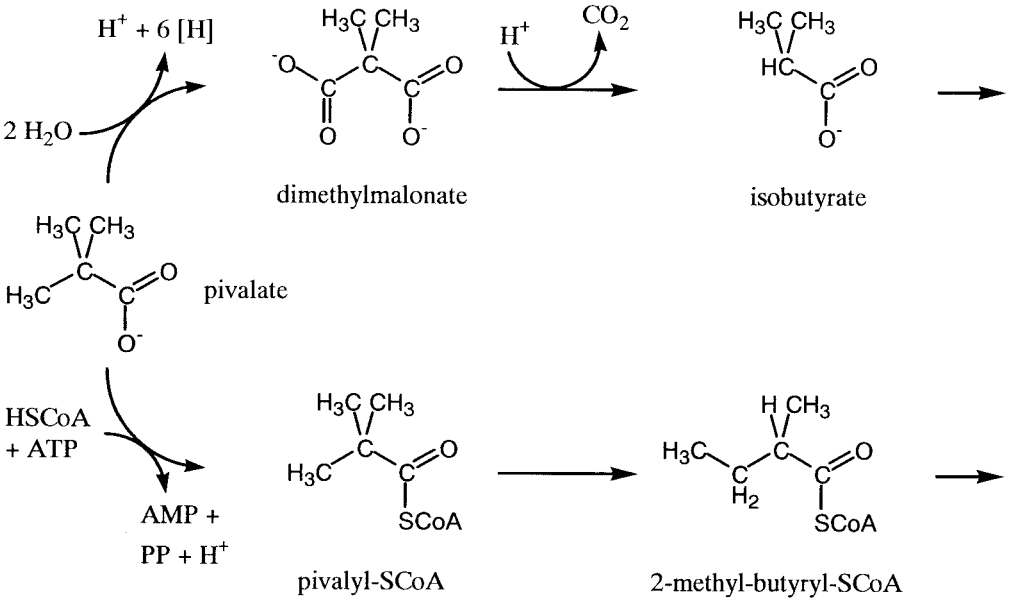

FIG. 3. Proposed intermediates for the degradation of pivalic acid.

$26\ H^+ + 26\ NO_3^- \rightarrow 25\ CO_2 + 13\ N_2 + 38\ H_2O$ requires the complete mineralization of 1.90 mM pivalate to balance the observed nitrate consumption. Additional experiments under a helium atmosphere showed the formation of 1.62 mol of N as dinitrogen and 0.57 mol of N as dinitrogen oxide from 2 mol of nitrate, indicating an incomplete reduction to dinitrogen. In prolonged incubation dinitrogen oxide disappeared. The complete mineralization of pivalic acid by strain PIV-1 was also supported by the pivalate-dependent carbon dioxide formation: the consumption of 1.92 mmol of C (pivalate) coincided with the formation of 1.13 mmol of carbon dioxide in the gas phase. This ratio was confirmed in pivalate-limited cultures. We observed nitrate but no nitrite or pivalate at the end of the incubation; the calculated pivalate dissimilation (based on denitrification) accounted for two-thirds of the pivalate consumption (data not shown).

Physiology of the isolated strains. Ascorbate was not utilized as carbon source. All strains grew on pivalate and nitrate in ascorbate chemically reduced medium. This excludes the participation of molecular oxygen as cosubstrate and of oxygenases in the mineralization pathway.

Simple organic acids with tertiary and quaternary carbon atoms were tested as growth substrates. Tertiary carbon atoms in α or β position, 2-ethylbutyrate or 3-methylbutyrate, were utilized by all strains. Strains PIV-3D, PIV-16-1, PIV-16-2, PIV-20-1, and PIV-34-1 did not grow on 2,2-dimethylbutyrate, consistent with an inhibition by the volume increase from a methyl to an ethyl group. The significance of the α position was revealed by the lack of growth and denitrification on 3,3-dimethylbutyrate, which has a quaternary carbon atom in β position to the carboxylic acid. Strain PIV-34-1 was the only strain able to grow on dimethylmalonate.

These growth tests on different substrates suggest the presence of at least two degradative pathways (Fig. 3). The utilization of pivalate and dimethylmalonate by strain PIV-34-1 hints at an anaerobic oxidation of a methyl group, similar to alkanes and toluene (2), and a degradation via dimethylmalonate. An alternative route is the activation to pivalyl-CoA and a rearrangement reaction yielding 2-methylbutyryl-CoA. The expected mutase is anticipated to belong to the family of coenzyme B_{12}-dependent mutases that includes methylmalonyl-CoA mutase and isobutyryl-CoA mutase (19). To our knowledge, such a mutase reaction on a quaternary carbon atom has never been reported. To test our hypothesis, future research will focus on the characterization of metabolites and the determination of enzyme activities.

Nucleotide sequence accession numbers. Gene sequences were deposited in the EMBL under accession numbers AJ505850 to AJ505863.

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